independent of the choice of agonist used to stimulate the system and should be identical to that observed in binding assays, providing that binding and response are studied under identical conditions. However, when an agonist mediates similar (or interacting) responses at different receptors, complications may arise; such complexities cannot yet be ruled out for the muscarinic subpopulations, and the use of Schild analysis to evaluate the possibility should be kept in mind (Furchgott, 1978). Finally, it should be pointed out that despite the difficulties associated with defining receptors according to agonist affinities (Kenakin, 1983), the relationship between the binding and response curves of agonists can be particularly useful in investigating the nature of receptor-effector coupling (Swillens and Dumont, 1980).

A large body of indirect evidence has been suggested to indicate that many muscarinic responses may be associated with the L agonist subpopulation (Birdsall et al, 1977; Strange et al, 1977; Fisher et al, 1983; Hanley and Iversen, 1978), but many of these studies are based on inter-tissue comparisons and all rely on agonist affinities. McKinney et al (1983) have recently reported that several agonists are much more potent in inhibiting adenylate cyclase than in stimulating guanylate cyclase in N1E-115 cells. Gil and Wolfe (1983) found the muscarinic stimulation of the turnover of PhI in parotid slices to be antagonized by lower concentrations of pirenzepine (16-fold) than were required to antagonize the muscarinic inhibition of adenylate cyclase in ventricular homogenates. The finding by the same authors that the classical antagonist atropine exhibited a 5-fold selectivity in the same direction illustrates the difficulty of such inter-tissue comparisons.

Nonetheless, the conclusions of McKinney et al (1983, above) and Gil and Wolfe (1983, above) are consistent in that if the adenylate cyclase response is attributed to the high affinity agonist subpopulation, the finding of Birdsall and Hulme (1983) that the high affinity subpopulation has low affinity for pirenzepine leads to the expectation that pirenzepine should antagonize the adenylate cyclase response with low affinity. These results are in accord with the suggestion that the muscarinic system may be similar to the α adrenergic system, in which α_1 receptors mediate calcium-related responses and α_2 receptors inhibit adenylate cyclase (Section IIIB). Physiological and biochemical studies (Hammer and Giachetti, 1982; Potter et al, 1984) have suggested that pirenzepine binds to "M_1" sites with high affinity in the forebrain and in ganglia and to "M_2" sites with low affinity in the brainstem, smooth muscle, and heart. The above tissue distributions are relative rather than absolute, but agree with the greater potency displayed by pirenzepine in antagonizing the stimulatory effects of muscarinic agonists in sympathetic ganglia, compared to its ability to block inhibitory muscarinic effects on the heart and smooth muscle (Hammer and Giachetti, 1982; Brown et al, 1981; Barlow et al, 1981).

A receptor (sub)classification based on a single antagonist (i.e., pirenzepine) faces a high risk that extra-receptor interactions may influence the classification. This problem emphasizes the importance of developing or discovering additional selective antagonists. As part of our interest in this area, we have examined studies in the literature concerning the antimuscarinic potencies of antidepressants and antipsychotics (Table 3). Potency in inhibiting the binding of {^{3}H}-QNB

was determined by Snyder et al (1974) in rat brain membranes, while potency in inhibiting the muscarinic stimulation of guanylate cyclase (cG_{50}) was determined by Richelson and Divinetz-Romero (1977) in neuroblastoma cells. Because of the marked inter-assay differences, the values are useful only by comparison between the classes of drugs or relative to the classical antimuscarinic, atropine. The two classes of drugs do not differ significantly by either of the individual measures (Q_{50}, cG_{50}), but the ratio of potencies in the two assays does separate the two groups. The antidepressants are relatively more potent in the binding assay, while the antipsychotics are more potent in the guanylate cyclase assay ($p < 0.001$, Table 3). One possible explanation for this finding is that the antipsychotics are selective for the subpopulation of receptors that is associated with the stimulation of guanylate cyclase, while the antidepressants are more potent at other sites. An alternative possibility is that the greater potency of the antipsychotics in the guanylate cyclase assay may be related to the inhibitory effects of phenothiazines and related compounds on calcium uptake in some systems (Hoss and Formaniak, 1984). To investigate the first possibility, we have examined the binding characteristics of some of the drugs in Table 3. The most interesting result to date is the similarity between the binding characteristics of clozapine and pirenzepine in the brainstem and forebrain (Table 4). There is a consistency between Tables 3 and 4 in that the pirenzepine-like selectivity of clozapine (Table 4) suggests that it should antagonize calcium-related responses with high affinity (see above), as it does (Table 3). However, it is also possible that clozapine and pirenzipine may have direct effects on calcium fluxes, due

to their structural similarities to the phenothiazines (see above). Such effects could complicate the evaluation of the relationship between subpopulation and response, because of the likelihood that calcium-related and calcium-independent responses may be mediated by different muscarinic subpopulations (above). Experiments are underway to test the extra-receptor influences of muscarinic ligands on calcium flux.

In summary, some consensus is developing in favor of the M_1, M_2 subclassification scheme, analogous to the α_1, α_2 adrenergic system, in which M_1 sites possess high affinity for pirenzepine and may be linked to calcium mobilization. As in the α_1 adrenergic system, there may be multiple agonist states for one or more of the antagonist sites (Birdsall et al, 1984). However, it must be remembered that neither this scheme nor several others that have been proposed are yet supported by well-developed pharmacologies, so that all must be considered tentative (Birdsall and Hulme, 1983).

V. Regulation of Muscarinic Receptors

A. Sensitization and Desensitization

Although some controversy remains, especially regarding denervation supersensitivity, there is now general agreement that the muscarinic receptor responds--at least under some conditions--in a manner similar to other CNS receptors, becoming sensitized after denervation of afferent pathways or chronic exposure to antagonists and becoming desensitized subsequent to stimulation or chronic agonist treatment. First considered here are results obtained with neuroblastoma-glioma hybrid cell lines.

In the neuron-derived cell lines, extended activation of the receptor with muscarinic agonists produces a severe loss (up to 88%) of muscarinic receptors (Klein et al, 1979), resulting in a concomitant decrease in the ability of muscarinic agonists to activate PI turnover (Siman and Klein, 1981) and inhibit adenylate cyclase (Klein et al, 1979). The agonist-induced decrease in the steady state number of receptors was owing to an increased rate of receptor degradation (Klein et al, 1979). Whereas antagonists inhibited the agonist-induced loss of receptors, they did not themselves directly affect receptor number. Inactivation of the muscarinic stimulation of cyclic-GMP formation also occurs, albeit on a shorter time scale, that is, minutes instead of hours (Taylor and Richelson, 1979). There is evidence that this short term effect on guanylate cyclase involves the activation-inactivation of calcium channels in the N1E-115 cell line (El-Fakahany and Richelson, 1980).

In the CNS, denervation of cholinergic pathways results in a modest, if any, increase in the number of muscarinic receptors in the target tissue. Thus, Westlind et al (1981) find a 20% increase in the dorsal hippocampus while Yamamura and Snyder (1974b) find no change in the whole hippocampus after septal lesions in rats. Lesion of the nucleus basalis produced a 28% increase in the high agonist affinity site without a significant change in B_{max} in rat cortex (McKinney and Coyle, 1982). These findings can be compared with data from peripheral tissues showing a lack of denervation supersensitivity in rat sympathetic ganglia (Burt, 1978a) and cat iris (Sachs et al, 1979), but a robust response in rat salivary gland (Pimoule et al, 1980).

In contrast to the denervation studies, a more consistent picture emerges from chronic treatments with cholinergic agonists and antagonists. Raising acetylcholine levels by chronic inhibition of acetylcholinesterase activity with di-isopropylfluorophosphate caused a 32% reduction in total muscarinic receptor level, owing to a 47% decrease in the low agonist affinity sites (McKinney and Coyle, 1982). These results corroborated the earlier findings of Gazit et al (1979) using Tetram, which is an organophosphate inhibitor of acetylcholinesterase.

Multiple infusions of carbamyl choline into the spinal cord of rats yielded a densensitization to the antinociceptive effect of the cholinergic agonist within 12 hr (Taylor et al, 1982). There was a concomitant loss of muscarinic receptors, reaching a value of 57% after the 12 hr period. Chronic intravenous infusion of oxotremorine in mice produced not only a tolerance to a variety of physiological and behavioral parameters, but also a loss of muscarinic receptors in several brain regions (Marks et al, 1981). It is also of interest that tolerance was observed at doses that did not cause a loss of receptors, suggesting that mechanisms other than receptor regulation are also operative in vivo. The observed loss of muscarinic receptors with chronic oxotremorine treatment was extended to developing animals by Ben-Barak et al (1981).

Chronic atropine treatment resulted in a doubling of the number of high agonist affinity sites, together with a smaller increase in low affinity sites (McKinney and Coyle, 1982). Likewise, chronic scopolamine treatment (10 mg/kg once daily for 10 days) leads to modest increases in total number of muscarinic receptors in several brain regions, both

during development and at maturity (Ben-Barak et al, 1981).

We have recently demonstrated a behavioral tolerance to low doses of scopolamine using a working memory task (Messer et al, 1983). Lesioning studies have demonstrated the importance of the septo-hippocampal pathway for this behavior (Thomas and Brito, 1980). Similar results are obtained whether scopolamine is administered systemically or directly into the hippocampus.

As shown in Fig. 3, animals drop to chance after the first injection, but develop tolerance to scopolamine within 2 days. In contrast to the other examples cited above for tolerance to muscarinic antagonists, there are no striking or apparent changes in muscarinic receptors, when they are examined in hippocampal homogenates after intraperitoneal injections (Table 5). Preliminary autoradiographic localization after intrahippocampal administration suggests that there may be some increase in receptor density, especially in the dentate gyrus (Fig. 4). Whether there are more dramatic localized changes in subtype distribution must await further experimentation and quantitation of the autoradiograms.

B. Hormones

Although the primary mode of interaction between cholinergic and other transmitter systems appears to be at the cellular level, for example, cholinergic input from the septum onto glutamate-containing granule cells in the dentate and dopaminergic synapses onto cholinergic interneurons in the caudate nucleus, there is also evidence for a direct interaction between muscarinic receptors and other transmitters or

neuromodulators occurring at the level of the plasma membrane. These effects include steroid hormones in the pituitary and brain, dopamine in the brain, and vasoactive intestinal peptide in the periphery.

Interestingly, the binding of tritiated classical antagonists in the adenohypophysis and preoptic area of the hypothalamus in rats and mice is heterogeneous, yielding curved Scatchard plots best fit by a two-site model (Avissar et al, 1981). Further, there is a sexual dimorphism in the binding characteristics, showing differences not only between males and females, but also among females at various stages of the estrous cycle.

A potent and specific effect of β-estradiol and progesterone was observed only in the hypothalmic and pituitary areas mentioned above, resulting in a decrease in both the proportion of high agonist affinity receptors and the value of the high affinity constant for oxotremorine (Sokolovsky et al, 1981). Antagonist binding was, however, unaffected by these hormones. These studies suggest an involvement of muscarinic receptors in the feedback mechanisms by which estrogens affect the release of gonadotropin.

Under certain ionic conditions, (50 mM Na/HEPES buffer containing 10 mM $MgCl_2$ and 10 mM Gpp(NH)p), the binding of {^{3}H}-QNB to muscarinic receptors in the striatum of rats was heterogeneous (Ehlert et al, 1981b). Apomorphine, and to a lesser extent dopamine and isoproternol, enhanced the binding of {^{3}H}-QNB by increasing the fraction of receptors in the high affinity antagonist form. The dopaminergic effect was inhibited by fluphenazine but not by haloperidol, suggesting that the effect was mediated by dopamine receptors of the D1 subtype.

There is now considerable support for the notion that vasoactive intestinal peptide (VIP), which is also found in the CNS (Giachetti et al, 1977), potentiates the action of acetylcholine in the periphery (see for example, Ahren and Lundquist, 1982). Evidence that VIP increases the affinity of acetylcholine, as well as other cholinergic ligands, for muscarinic receptors in the cat salivary gland has been obtained by Lundberg et al (1982).

C. Copper-Deficient Animals

Among the metals that were shown to affect muscarinic receptors in vitro, Cu^{2+} was highly potent, showing effects well within the physiological range of Cu concentration in brain (see Section IIF above). In order to assess a possible role for Cu in vivo, the receptor was characterized in rats made Cu deficient by a dietary regimen (Farrar and Hoss, unpublished). As shown in Table 6, in forebrain regions there was a decrease in both the affinity of the receptors for {^{3}H}-1-QNB and the density of receptors in the Cu-deficient animals compared with control animals. Cu treatment in vitro of homogenates from deficient animals did not reverse the in vivo effects but rather decreased receptor occupancy and ligand affinity in a manner similar to Cu treatment of control homogenates. Interestingly, minimally deficient rats displayed very similar changes in receptor properties compared with more severely deficient animals. Minimal Cu deficiency produced robust effects on the binding of agonists, increasing IC_{50} and derived dissociation constants values (Table 7). The addition of Cu to the assay medium caused an apparent reversal of the in vivo Cu deficiency, decreasing IC_{50} and

derived dissociation constants to values near those observed with homogenates from normal animals in the presence of Cu. In summary, Cu deficiency has dramatic effects on both receptor number and the binding of agonists to muscarinic receptors in the CNS, suggesting that Cu may have an endogeneous role in the regulation of the receptor. Since Cu^{2+} is a sulfhydryl ligand with a propensity for forming stable square planar complexes, perhaps Cu participates in the dimer-tetramer equilibrium corresponding to low and high agonist affinity data, respectively, as suggested by Avissar and her associates (Avissar et al, 1983).

D. Protein Phosphorylation

Burgoyne (1980,1981,1983; Burgoyne and Pearce, 1981) has presented a variety of evidence that phosphorylation of proteins inhibits the specific binding of the muscarinic antagonist{ ^{3}H}-QNB by a reduction in receptor number. Further, the reduction in muscarinic receptors that occurs when primary cerebellar cultures are incubated with cholinergic agonists is accompanied by an increase in phosphorylation of several membrane proteins, one of which has a molecular weight similar to that of the component that binds the irreversible muscarinic antagonist PrBCM (Burgoyne and Pearce, 1981). In studies of membranes from rat brain, Burgoyne also concluded that subpopulations of muscarinic receptors, defined by affinities for agonists, were differentially regulated by phosphorylation (Burgoyne, 1983). It is quite likely that muscarinic receptors are involved in the regulation of the phospholipid-dependent kinase (C-kinase) described by Nishizuka and his associates (Nishizuka, 1983; see Section IIIA). However, the possibility that C-kinase may be

involved in the phosphorylation-induced decrease in muscarinic receptors has not been investigated to date. As described above (Section VA), down-regulation of muscarinic receptors, due to continuous agonist occupancy, appears to occur in two phases. In the first phase, a decrease in the ability to elicit a response (Richelson and El-Fakahanay, 1981; Burgoyne and Pearce, 1981) occurs over a period of minutes, while receptor binding sites are unaltered. Then, over a period of hours, the number of binding sites decreases (Shifrin and Klein, 1980; Gazit et al, 1979; Burgoyne and Pearce, 1981). This loss in binding capacity is accompanied by a decrease in the binding affinity of the agonist carbachol, with a concomitant loss in the ability of guanyl nucleotides to shift the agonist binding curve toward lower affinity (Chin et al, 1980). Although speculative, it is possible to accommodate much of the data regarding phosphorylation and down-regulation by postulating that phosphorylation "marks" the receptors for degradation or for some other irreversible alteration (possibly internalization). If the action of chronic agonist occupancy is envisioned as favoring kinase activity over an ongoing phosphatase activity, such an action would enhance the probability of degradation. This postulate would explain the findings that: (a) the effects of phosphorylation on receptor loss are not reversed by phosphatase (Burgoyne, 1983); (b) peptides that are phosphorylated with a time course similar to that of receptor loss are smaller than the "intact" receptor (Burgoyne and Pearce, 1981); and (c) the return of receptor density to normal levels in culture, following removal of agonist, requires protein synthesis (Shifrin and Klein, 1980). Additionally, it would be possible that the phosphorylation per se might

be responsible for short term desensitization, as reported for the luteinizing hormone (Hunzicker-Dunn et al, 1979) and β adrenergic (Stadel et al, 1983) receptors. Soklolovsky (1984) has recently suggested that protein phosphorylation may play a role in the dimerization of muscarinic receptors (Section IIG).

VI. Solubilization of Muscarinic Receptors

Successful solubilization of the receptor from brain has been achieved using digitonin (see for example, Aronstam et al, 1978b), sodium cholate/sodium chloride (Carson, 1982), and the zwitterionic detergent CHAPS (Gavish and Sokolovsky, 1982). Increased stability of the solubilized receptor was realized using glycerol with digitonin (Baron, 1982) and lecithin with sodium cholate/sodium chloride (Hulme et al, 1983). In general, the solubilized receptors are homogeneous with respect to the binding of classical antagonists. Whereas digitonin-solubilized receptors from brain are also homogeneous with respect to agonist binding (Hurko, 1978; Baron, 1982), CHAPS-solubilized receptors may be heterogeneous as evidenced by flattened binding curves (Gavish and Sokolovsky, 1982), although these authors do not mention this point. Agonist binding to receptors solubilized with CHAPS is, moreover, decreased by GTP in a Na^+-dependent manner (Kuno et al, 1983). It is also of interest that digitonin-solubilized receptors retain their response to low concentrations of Cu^{2+} (Baron, 1982), suggesting perhaps that the low affinity agonist form of the receptor has been preferentially solubilized.

VII. Concluding Remarks

There is now compelling evidence that binding sites defined using tritiated classical muscarinic antagonists such as QNB represent physiological receptors that mediate many of the actions of acetylcholine in brain as well as other tissues. That agonists and non-classical antagonists distinguish different populations of the receptor on the basis of affinity is equally apparent. Studies of the binding of radioligands to homogenized tissue, together with autoradiographic analyses of brain sections are providing detailed maps of muscarinic receptor distribution throughout the brain. The recent achievement of the first image of the receptor in a living person using computed tomography after injection of 4-(^{123}I)-iodoQNB (Eckelman et al, 1984) raises hope for the clinical assessment of the state of CNS muscarinic receptors in various neuropathological conditions.

Muscarinic receptors are ubiquitous in the brain, in terms of receptor density and of their widespread involvement in behavioral phenomena (Karczmar, 1977). However, it is presently difficult to analyze specifically the cholinergic components of these processes, due to the diffuse nature of the cholinergic system. That is, cholinergic agents have such diverse effects that it is difficult to examine specific functions in isolation. This feature underscores the importance of the cholinergic system, but severely limits its experimental and therapeutic utility. For example, muscarinic antagonists continue to have a place in the treatment of Parkinson's disease, usually due to adverse effects associated with L-DOPA therapy (Weiner, 1982). The nonselective

antimuscarinics that are currently available exacerbate the dementia that is often associated with Parkinson's disease (Barbeau, 1980; Ruberg et al, 1982). It is possible that the therapeutic and adverse effects may be controlled by independent subpopulations of muscarinic receptors, and that the development of drugs with greater selectivity toward the muscarinic subpopulations will prove beneficial in this and other neurological disorders.

However, the number and functions of the various subpopulations distinguished on the basis of differential affinities of some muscarinic ligands remains unclear. Two current approaches--development of specific ligands, especially antagonists, and identification of the effector systems linked to each receptor subtype--hold promise for unraveling this complex system. The importance of carrying out binding studies under conditions identical to those used for biochemical assays requires attention if correlations between receptor occupancy and function are to be meaningful.

There may be an advantage in studying responses that are closely linked to agonist occupancy. In this regard, muscarinic stimulation of the breakdown of inosital phospholipids can be readily investigated in brain (Downes, 1982); recently, muscarinic and opiate-specific GTPase activities, presumably associated with the regulation of adenylate cyclase, have also been demonstrated in brain (Franklin and Hoss, 1984; Onali et al, 1983).

The studies of Fisher et al (1980a) have indicated that the use of rigid antagonists of low affinity may be the key to detecting subtle differences in receptor structure or conformation. Although receptor

subtypes are best defined according to the affinities of selective, competitive, antagonists, events occurring beyond receptor occupation may contribute to the empirical selectivity of agonists. Thus, an agonist of low efficacy may initiate a full-blown response at a synapse that exhibits a large receptor reserve, but mainly antagonist properties where a receptor reserve is lacking. Such coupling phenomena probably explain muscarinic agonist-antagonist properties that have been reported recently in brain (Fisher et al, 1983; Nordstrom et al, 1983). The allosteric site that seems to bind gallamine and the agonist McN-A-343 may offer another means to "adjust the gain" between muscarinic binding and response.

The synaptic environment continually subjects muscarinic receptors to the dynamic processes of sensitization/densensitization, up-/down-regulation, and coupling/uncoupling. It seems likely that these regulatory mechanisms contribute to the development of tolerance and may complicate long-term drug regimens. The regulation of muscarinic receptors by transition metals, sulfhydryl reagents, and protein phosphorylation may provide insight into the molecular mechanisms involved in these processes. Subpopulations of muscarinic receptors may be regulated differentially (Smit et al, 1980; Chin et al, 1980; Burgoyne, 1983; Farrar and Hoss, 1984). The development of ligands with greater subpopulation selectivities, coupled with recent advances in affinity labeling and the solubilization of active receptors, can be expected to clarify further the interrelationships among muscarinic subpopulations.

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TABLE 1. Effects of gallamine on the binding kinetics of {^{3}H}-NMS and {^{3}H}-QNB to rat brain membranes

	Association	
	$t_{\frac{1}{2}}$[a] (min)	% inhibition[b]
^{3}H NMS (1 nM)	0.52 ± 0.10	---
+ 3 μM gallamine	4.6 ± 0.35	36%
^{3}H QNB (0.5 nM)	3.9 ± 0.4	---
+ 15 μM gallamine	4.5 ± 0.7	58%
^{3}H QNB (5 nM)	0.28 ± .04	---
+ 100 μM gallamine	0.17 ± .08	47%

	Dissociation[a,c]
	$t_{\frac{1}{2}}$
^{3}H NMS + QNB	12.2 ± 0.5 min
+ QNB + gallamine (100 μM)	87 ± 4 min
^{3}H QNB + QNB	10.8 ± 0.4 hr
+ QNB + gallamine (15 μM)	11.3 ± 0.5 hr
+ QNB + gallamine (100 μM)	11.7 ± 0.4 hr

a half times ($t_{\frac{1}{2}}$) were determined by monoexponential fits

b % inhibition was calculated at 10 min for 5 nM {^{3}H}-QNB and at 30 min for the other cases.

c dissociation was initiated by the addition of 1 μM unlabeled QNB, with or without gallamine, as indicated.

TABLE 2. Effect of Carbachol on the Incorporation of ^{32}P into PhI.

Conditions	CPM per nmol inorganic* phosphate in PhS/PhI spot
Control	12.8
+10 mM carbachol	28.5
+ 1 M scopolamine	13.5
+10 mM carbachol + 1 M scopolamine	12.3
+ 1 mM gallamine	13.4

* The labeling of phosphatidylserine (PhS) is not affected by carbachol, so that the increase seen is due to PhI alone. The values shown are the averages of quadruplicate determinations. The SEM values were within 10% of the means. Experimental details are given in the legend to Fig. 2.

TABLE 3. Antimuscarinic potencies of various agents, expressed as the concentrations which inhibit the muscarinically stimulated formation of cGMP (cG_{50}) or the binding of {^3H}-QNB (Q_{50}) by 50%.

	cG_{50}(nM)	Q_{50}(nM)	Ratio(a/b)*
Antimuscarinics			
QNB		0.3	
atropine	2	0.4	5
scopolamine			
Antidepressants			
amitriptyline	100	10	10
doxepine	300	44	6.8
imipramine	400	78	5.1
nortriptyline	1000	57	18
desipramine	2000	170	12
Antipsychotics			
clozapine	3	26	0.115
thioridazine	60	150	0.4
promazine	2000	650	3.1
chlorpromazine	2000	1000	2
fluphenazine	2000	12000	0.17
perphenazine	4000	11000	0.36
acetophenazine	4000	10000	0.4
haloperidol	7000	48000	0.15
trifluoperazine	20000	13000	1.5
	**p > 0.1	**p > 0.1	**p > 0.001

* The ratio cG_{50}/Q_{50} may reflect the selectivity of these drugs toward muscarinic subpopulations. To account for differences between the assays, atropine may be taken for comparison as an antagonist which does not distinguish subpopulations. The data are from the literature: (a) Richelson and Divinetz-Romero (1978), neuroblastoma cells; (b) Snyder et al (1974), rat brain membranes.

** two-tailed t-tests for differences between antipsychotic and antidepressant values.

TABLE 4. Binding properties of typical and atypical muscarinic ligands[a,b].

	FOREBRAIN		BRAINSTEM	
COMPOUND	n_H	pIC_{50}	pIC_{50}	n_H
atropine	0.94	8.6	8.9	0.96
N-methylscopolamine	1.02	9.4	9.4	0.97
carbachol	0.46	5.5	7.0	0.44
pirenzepine	0.68	7.0	6.2	0.98
clozapine	0.70	7.6	6.8	0.92

a) The binding of the unlabeled ligands to crude synaptic membrane preparations was determined by competition for 0.2 nM {^{3}H}-NMS by previously described methods (Ellis and Hoss, 1980).

b) Results are averages of 2-6 experiments.

TABLE 5. Effect of Daily Scopolamine Injections on Muscarinic Receptors in the Hippocampus of Trained Rats.

	Experimentals	Controls
QNB:		
B_{max}	2.36±0.27 pmole/mg	2.00±0.27 pmole/mg protein
K_d	$(5.31\pm0.96)\times10^{-11}$ M	$(5.03\pm1.6)\times10^{-11}$ M
Carbachol:		
IC_{50}	$(4.33\pm0.37)\times10^{-5}$ M	$(3.10\pm1.0)\times10^{-5}$ M
α	0.280±0.048	0.301±0.019
K_H	$(8.05\pm3.5)\times10^{-8}$ M	$(7.27\pm2.6)\times10^{-8}$ M
K_L	$(4.19\pm1.1)\times10^{-5}$ M	$(4.21\pm0.90)\times10^{-5}$ M

Values are means ± SEM of 5-6 experiments each performed in triplicate. Experimental details are given in the legend to Fig. 4.

TABLE 6. Effect of Dietary Cu Deficiency on Receptor Number and K_d for QNB in Rat Forebrain[a].

Animal	Receptor Number (pmole/mg protein)	K_d (x10-10 M)	Cu[b] (µg/g dry wt.)
Control	2.26 ± 0.02	0.62 ± 0.04	17.0 ± 0.94
Deficient	1.50 ± 0.05	1.07 ± 0.12	5.6 ± 0.42
Minimally Deficient	1.60 ± 0.00	0.91 ± 0.14	12.8 ± 0.95

[a] Experimental details are similar to those given in the legend to Fig. 4. Values are the means ± SEM of 3-4 experiments each performed in triplicate.

[b] Data shown for cortex.

TABLE 7. Effect of Dietary Cu Deficiency on the Binding of Carbamyl Choline to Muscarinic Receptors in Rat Forebrain.

Animal	IC_{50} (μM)	K_H (μM)	K_L	α
Control	5.44 ± 0.20	0.22 ± 0.05	41.0 ± 6.9	0.41 ± 0.01
Minimally Deficient	29.8 ± 3.0	0.48 ± 0.02	118 ± 8.2	0.41 ± 0.02

Experimental details are similar to those given in the legend to Fig. 4. Values are the means ± SEM of 3-4 experiments each performed in triplicate.

LEGENDS TO FIGURES

Fig. 1 The effect of increasing medium Cu on the displacement of 50 pM {^{3}H}-QNB by 5 μM and 1 μM carbamylcholine in forebrain (●) and brainstem (▲) preparations, respectively. The reduction in % QNB bound (% B) indicates increased carbamylcholine binding. The data represent the means of three experiments each performed in triplicate.

Fig. 2 Comparison of carbachol-stimulated PhI turnover to the occupancy curve for carbachol.

Tissue slices (150 μm x 150 μm) were prepared from the forebrains of male Sprague-Dawley rats and incubated in Krebs-Ringer bicarbonate buffer (KRB). The incorporation of {^{32}P}Pi into PhI in a period of 45 min (37°C) was measured by extracting the phospholipids (Folch et al, 1957), separating them by thin layer chromatography (Skipsky et al, 1964), and scraping the PhI region of the plate. Radioactivity was determined by Cerenkov counting and Pi was determined later, as described by Plesums and Bunch (1971). Binding assays were performed with identical slices under identical conditions, by competition for the specific binding of 0.5 nM {^{3}H}-NMS. The affinity of {^{3}H}-NMS was 0.83 nM (single-site kinetics). The dose-response curve for the PhI effect (Δ) is shown superimposed on the occupancy curve for carbachol (●) and mass-action binding curves from a two-site fit of the binding data (41% high affinity sites). All curves were drawn by computer

and the response curve is based on a one-site model, EC_{50} = 37 μM.

Analysis of the curves was performed by previously described methods (Ellis and Hoss, 1982).

Fig. 3 Effect of scopolamine on representational memory in a T maze. Male hooded rats of the Long-Evans strain were trained to perform a non-matching to sample task in a T-maze. After rats demonstrated proficiency (100% correct for three consecutive sessions), they were divided into two groups, one receiving 2 mg/kg daily i.p. injections of scopolamine (●) and the other receiving saline vehicle (0) 15 min before testing.

Fig. 4 Binding of QNB and carbachol to muscarinic receptors from the hippocampi of trained rats.

A. Scatchard plot used to estimate the values of K_d and B_{max} for the specific binding of {^{3}H}-1-QNB where b and f refer to bound and free QNB, respectively. Least squares analysis gave B_{max} = 2.43 pmole/mg protein and K_d = 3.53 x 10^{-11} M.

B. The inhibition (i) of QNB binding by various concentrations of carbachol (CCh). The symbols represent the experimental values and the line is calculated from the best fit to a two-site model. Data analysis as described elsewhere (Ellis and Hoss, 1980) gave IC_{50} = 4.96 x 10^{-5} M, K_H = 7.45 x 10^{-8} M, K_L = 5.80 x 10^{-5} M and α (fraction of receptors with high affinity) = 0.32.

The binding assays were performed on a washed membrane fraction prepared from the hippocampi of trained rats sacrificed 24 hr

after the last injection (see legend to Fig. 3).

Fig. 5 Autoradiographic localization of muscarinic receptors in trained rats. Rats were training as described in the legend to Fig. 3. Scopolamine (A) 30 μg in 0.5 μl or saline (B) was injected bilaterally through guide cannulae aimed at the CA3 region of each hippocampus four times over a period of 12 days. Brains were lightly fixed by perfusion with 0.1% formaldehyde in phosphate buffer 24 hr following the last injection and 24 μm coronal sections cut on a cryostat microtome. The sections, which were mounted on microscope slides, were incubated with 4 nM {^{3}H}-1-QNB for 1 hr and rinsed twice with buffer. The slides were subsequently exposed to X-ray film for 5 days at 4° C. The prints were made using the autoradiograms as negatives. Nonspecific binding, which was evaluated by including excess atropine with adjacent sections, was virtually absent. Scatchard analysis indicated that the total specific binding per section (80 fmole) was the same for both animals.

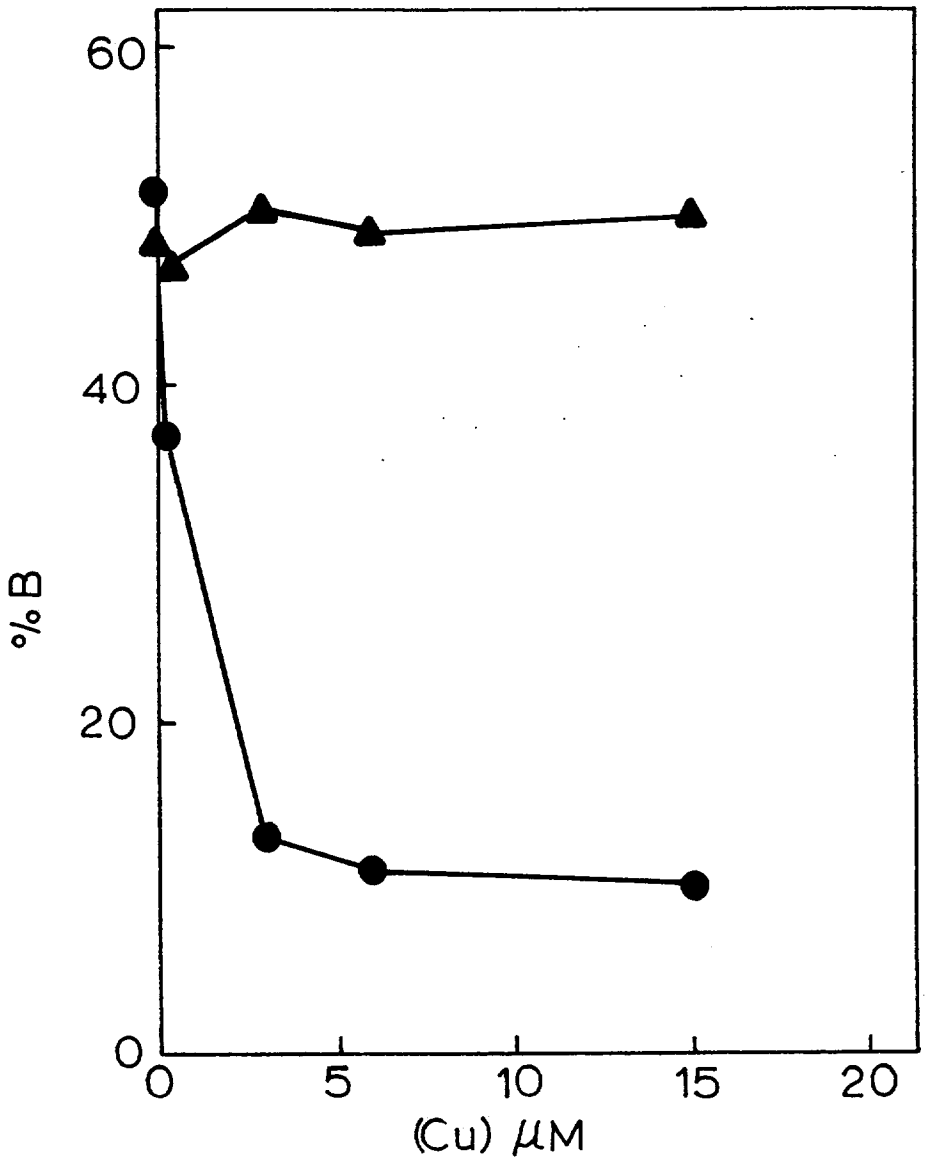


Fig. 1

COMPARISON OF BINDING DATA TO RESPONSE DATA

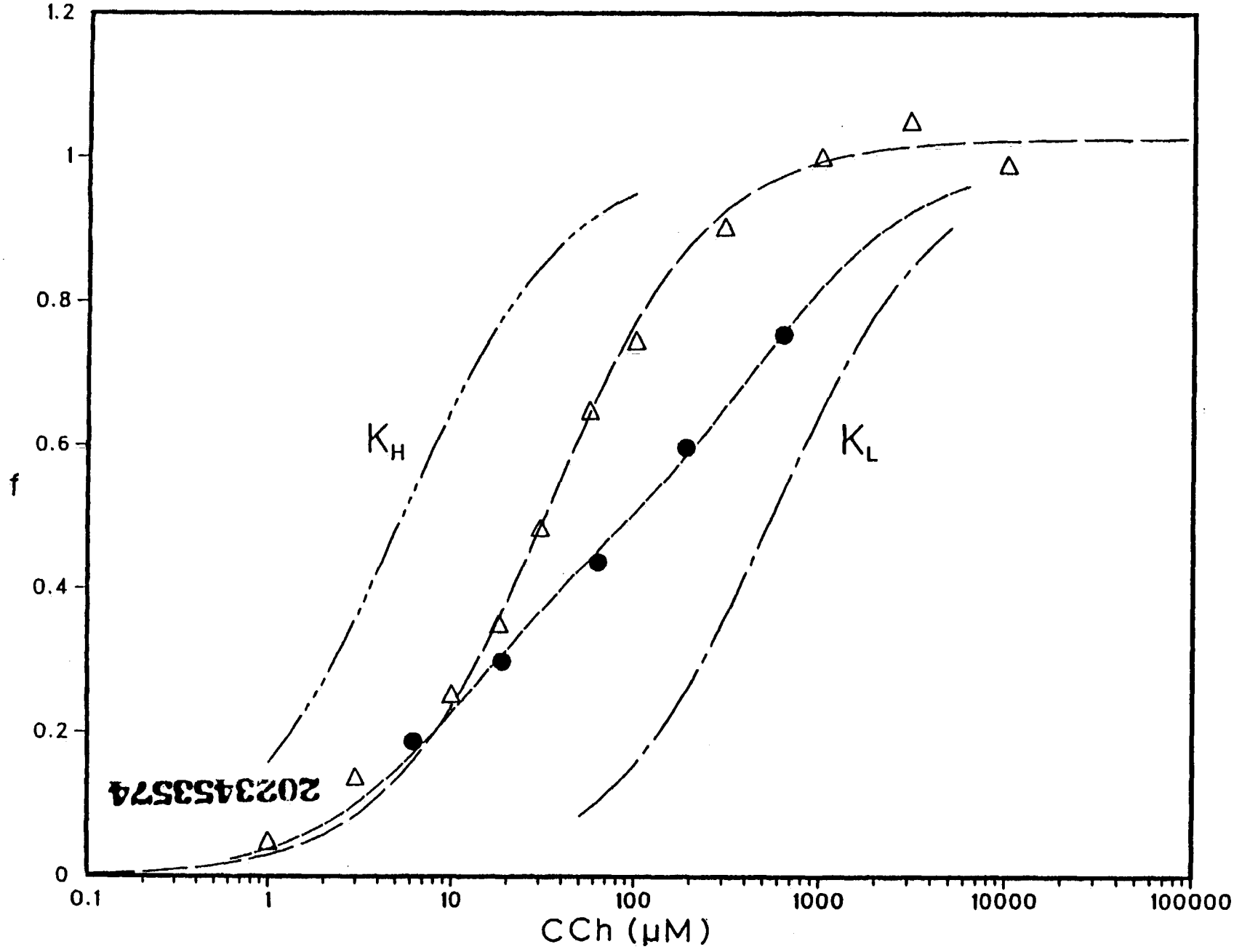
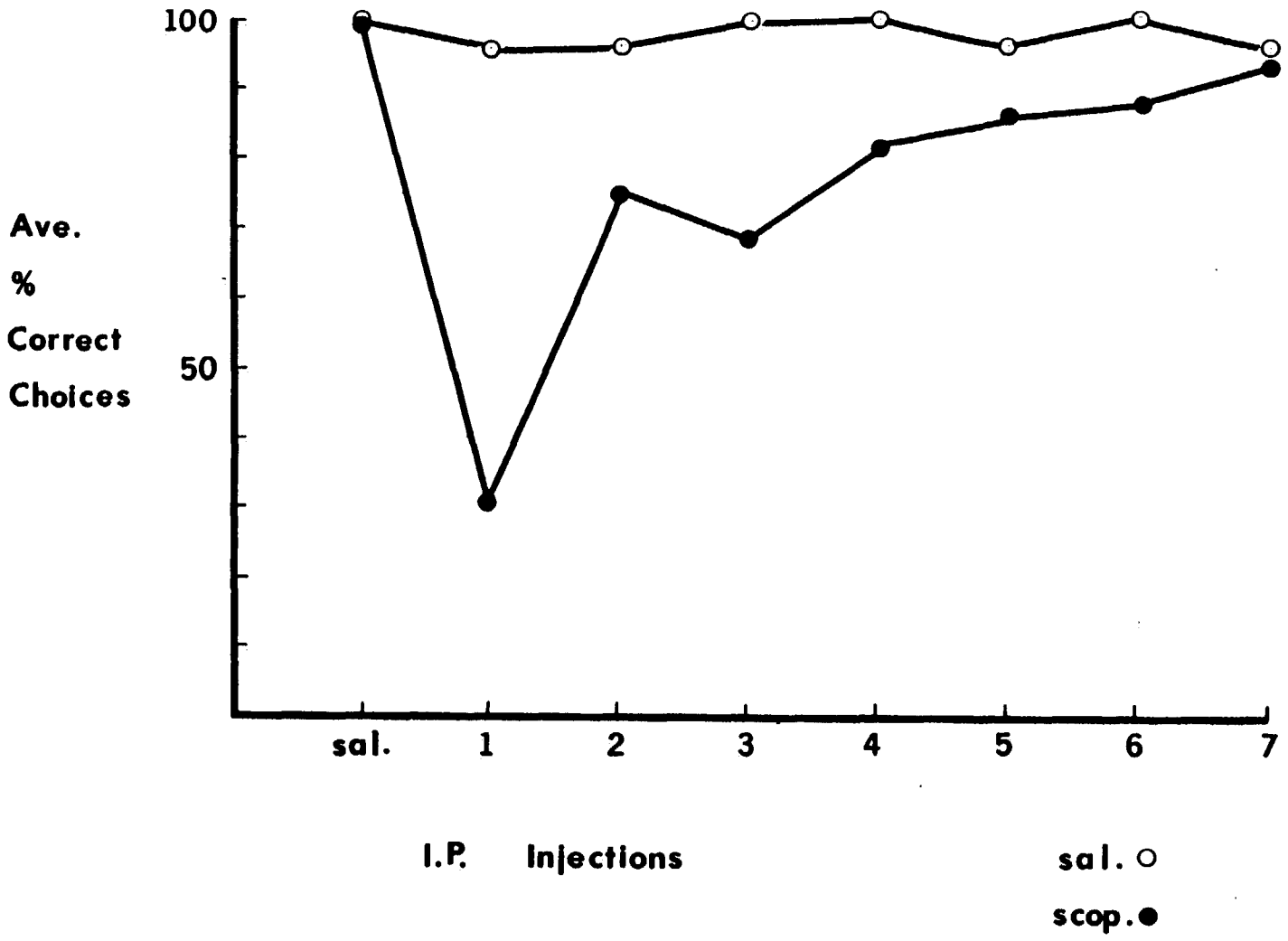
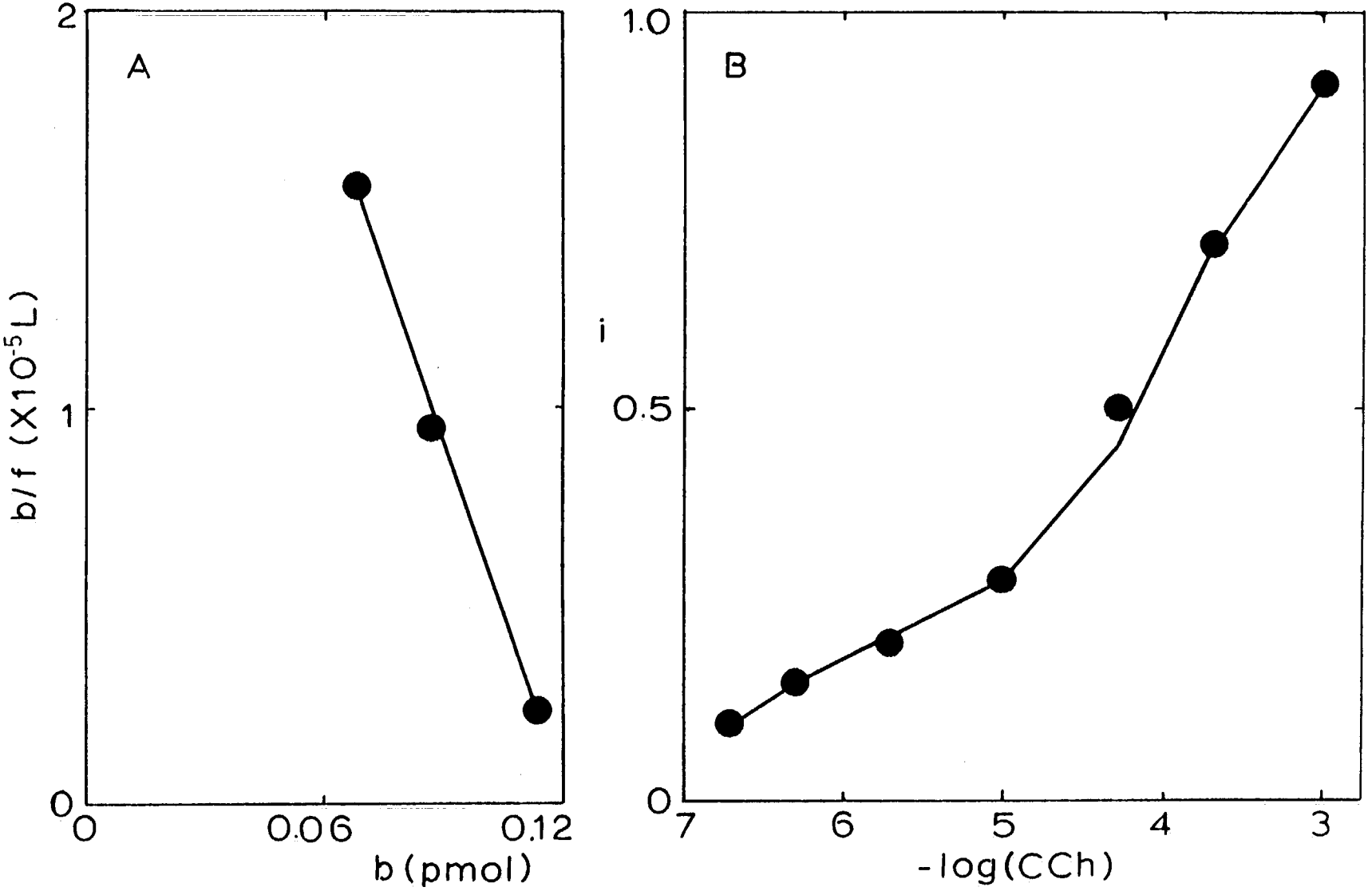


Fig. 2





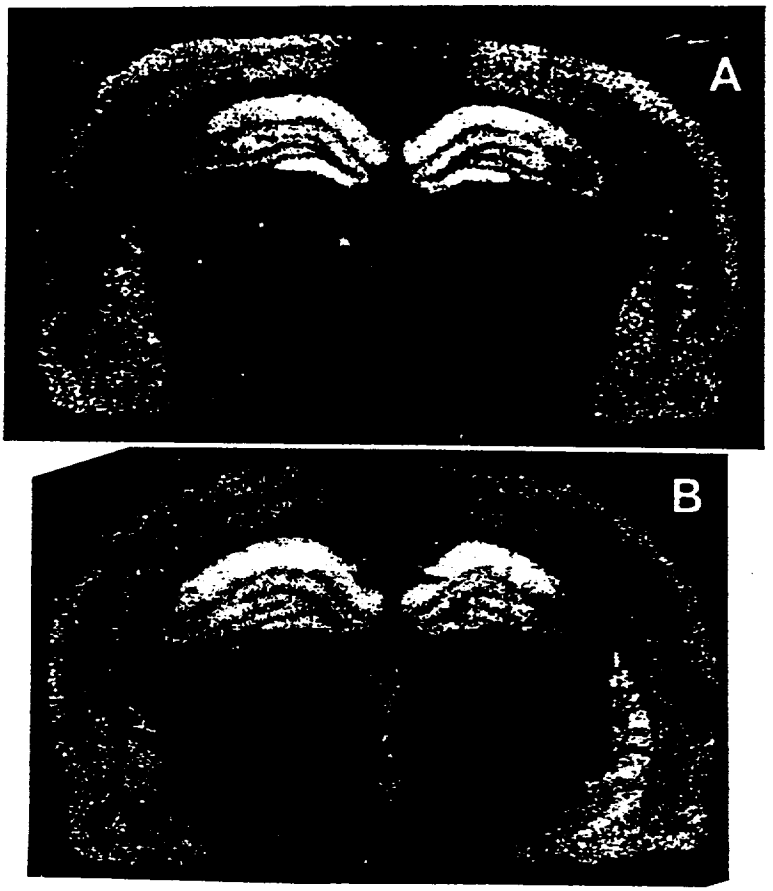


Fig. 5 Hoss & Ellis